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Comparative biotoxicity of N-Phenyl-1-naphthylamine and N-Phenyl-2-naphthylamine on cyanobacteria *Microcystis aeruginosa*

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HIGHLIGHTS

- P_1NA and P_2NA with similar molecular structure, only P_1NA was found high toxic to *M. aeruginosa.*
- ROS induced by P_1NA could the reason of the growth inhibition of *M. aeruginosa.*
- The formation of 1,4-naphthoquinone was the key factor to generate excessive ROS.

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ABSTRACT

N-Phenyl-1-naphthylamine (P_1NA) and N-Phenyl-2-naphthylamine (P_2NA) are both widely used as antioxidant and plant secondary metabolites. In this study, growth, esterase, photosynthetic activity and cell membrane integrity were used as biomarkers to compare biotoxicity of P_1NA and P_2NA on *Microcystis aeruginosa*. According to the results, a dose-response relationship was observed only between P_1NA concentrations and growth inhibition. The EC_{50} (48 h) of P_1NA calculated from growth inhibition was 16.62 μM, while that of P_2NA was not detected. When the esterase and photosynthetic activity were applied to evaluate the biotoxicity, it was found that a concentration of 20 μM P_1NA, P_2NA caused reduction of esterase activity and Fv/Fm of *M. aeruginosa* to 22.2 and 3.3%, 97.5 and 92.1%, respectively, after 48 h exposure. The percentage of membrane-damaged cells was increased as P_1NA exposure concentration increased, but that was not detected when exposure to P_2NA. The difference substituted position in the molecular structure of P_1NA and P_2NA leads to different toxicological properties and only P_1NA was found highly toxic to *M. aeruginosa*. The toxicity is due to that only P_1NA can be biotransformed to 1,4-naphthoquinone, which could induce overproduction of intracellular ROS as well as result in oxidative damage and growth inhibition of test organism.

1. Introduction

N-Phenyl-1-naphthylamine (P_1NA) serves as an antioxidant in various lubrication oils and also as a protective agent in rubbers as well as rubber mixtures. (Koennecker et al., 1998). It was estimated that the annual production of P_1NA was 5800–6300 tons from 1986 to 1990 in the world (Koennecker et al., 1998). The distribution of P_1NA was predicted approximately 29% to water, 34% to sediment, 36% to soil, and less than 1% each to air, suspended sediment, and biota in the environment using a Level II fugacity model (Mackay and Models, 2001; Koennecker et al., 1998). N-Phenyl-2-naphthylamine (P_2NA), the isomer of P_1NA, also used as an additive jet oils and it is a byproduct in the manufacture of P_1NA (Winder and Balouet, 2002). It was reported that P_1NA was detected in river freshwater (2–7 μg L^{-1}) and sediment (1–5 mg kg^{-1}) near a small specialty chemicals manufacturing (Koennecker et al., 1998). And P_2NA has been detected in sediments and freshwater in the proximity of chemical production sites in mg kg^{-1} and μg L^{-1} concentrations (Brack et al., 1999; Altenburger et al., 2006).

Studies performed according to standard protocols yielded LD_{50} of P_1NA in male and female Wistar rats of >5000 mg kg^{-1} body weight (Koennecker et al., 1998). Acute oral toxicity test of P_2NA

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indicated that its LD_{50} in rats and in mice were 8730 mg kg^{-1} and 1450 mg kg^{-1} body weight (NTP, 1988). Due to P_1NA and P_2NA's lipophilicity with octanol-water partition coefficient, log K_{OW} of 4.2 and 4.38, respectively, they may be expected to have a considerable bio-accumulative potential in aquatic environments and be toxic to aquatic organisms. The studies from laboratory tests with ciliates, *Daphnia*, and fish indicated that P_1NA was highly toxic to aquatic species. EC_{50} (48 h) for the inhibition of cell proliferation of freshwater ciliates (*Tetrahymena pyriformis*), LC_{50} (48 h) with young *Daphnia magna* and LC_{50} (96 h) in a flow-through system for rainbow trout (*Oncorhynchus mykiss*) are, respectively, 2 mg L^{-1} (Epstein et al., 1967), 0.30 mg L^{-1}, and 0.44–0.74 mg L^{-1} (Sikka et al., 1981). Phytoplankton is the fundamental part in the aquatic ecosystems. However, there is little research about the toxicity of P_1NA on phytoplankton.

Submerged plants can secrete secondary metabolites to inhibit photosynthetic plankton in order to compete for light and nutrition dominance in the water environment. This phenomenon is called allelopathy and the active substances are known as allelochemicals (Rice, 1984). In recent years, significant progress in the allelopathic effect of submerged macrophyte on cyanobacteria has been made in both laboratory and field study. In respect of the mechanism of allelopathy, it was reported that aquatic plants release allelochemicals continually with low concentration, and caused persistent stress on target organisms. Under this condition, the intracellular signaling radicals (ROS, NO) were induced, as well as, the programmed cell death (PCD) was trigger in *M. aeruginosa* which was confirmed by the hallmarks of PCD, caspase-3-like protease activity, DNA fragmentation, and destruction of cell ultrastructure (He et al., 2016). The engineering application of allelopathy through reconstructing the aquatic vegetation in the restoration of the subtropical eutrophic lake was studied. Ma et al. (2009) reported that transformation of water in Yuehu Lake (66 ha), a shallow eutrophic lake in China, from turbid algae green to a transparent state, was achieved by restoring the submerged macrophyte community while the nitrogen and phosphorus are at the level of eutrophication. Thus, it attracted the great interest of researchers to study the mechanism of allelopathy. P_1NA and P_2NA as a kind of allelochemicals have been isolated and identified in axenic as well as noaxenic root exudates of *Eichhornia crassipes* (Sultankhodzhaev and Tadzhibaev, 1976; Sun et al., 1993). These two substances have also been detected in the culture medium of three submerged macrophytes (*Elodea nuttallii*, *Hydrilla verticillata* and *Vallisneria spiralis*) by GC-MS (Gao, 2010). Qian et al. (2009, 2010) studied the toxicity of allelochemical P_2NA to *Chlorella vulgaris* and *M. aeruginosa*. The only difference in molecular structure between P_1NA and P_2NA is that anilino-N substitutes at 1 and 2 positions of the naphthyl group. No information is available about whether the toxicity of P_1NA and P_2NA on cyanobacteria is different and how the toxicity is produced.

The present study, *M. aeruginosa*, a dominant species of cyanobacterial blooming, was chosen as a target species. The physiological and photosynthetic parameters i.e. growth inhibition, esterase activity, cell membrane integrity, and photosynthetic activity were used as biomarkers to compare the toxicity between P_1NA and P_2NA. Moreover, reactive oxygen specious (ROS) level was used to evaluate the toxicity mechanism. The aim of this study was to (1) provide ecotoxicological information for evaluating the toxicity of P_1NA and P_2NA to the cyanobacteria (*M. aeruginosa*) in future ecological risk assessments and regulation of P_1NA and P_2NA use and discharges in aquatic ecosystems, (2) elucidate toxicity and inhibition mechanism of P_1NA and P_2NA on cyanobacteria to provide the base for further study the mechanism of allelopathic mechanism.

2. Materials and methods

2.1. Cultures conditions

Cyanobacteria, *Microcystis aeruginosa* FACHB 905 was provided by the Institute of Hydrobiology of the Chinese Academy of Sciences. *M. aeruginosa* was cultured in autoclaved BG11 media (Rippka et al., 1979) at 25 ± 0.5 °C in an incubator under a light intensity of 18 μmol m^{-2} s^{-1} with a 12:12 h light: dark cycle. The cultures were shaken three times per day by hands.

2.2. Chemicals

N-phenyl-1-naphthylamine (98.0%) and N-phenyl-2-naphthylamine (97.0%) were commercially obtained from the same company (TCI, Tokyo, Japan). 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), fluorescein diacetate (FDA) and dimethyl sulfoxide (DMSO, 99%) were all obtained from Sigma (St. Louis, Missouri, USA). Propidium Iodide (PI) was purchased from Sangon Co. Ltd (shanghai, China).

2.3. Toxicity assays

2.3.1. Experimental design

Conical flasks (250 mL) and culture medium were prepared and autoclaved. 100 mL culture media was added to each bottle. P_1NA and P_2NA were added with the concentration of 0, 5, 10, 15, 20 μM, respectively. Then *M. aeruginosa* was inoculated into each flask with the initial density of 1×10^6 cells mL^{-1} when it was at the exponential growth phase. The samples were taken after 48 and 72 h for the measurement of growth and other physiological parameters. The stock solutions of P_1NA and P_2NA were prepared with DMSO which in test solution was less than 0.1% (v/v). 0.1% DMSO had no effects on the growth of *M. aeruginosa* (FACHB-905) (Gao et al., 2011). The experimental conditions are the same to the cyanobacteria culture conditions shown above. All the experiments were performed in triplicate.

2.3.2. Measurement of growth inhibition

The cells density was measured by spectrophotometry at 680 nm. The regression equation between cell density ($y \times 10^3$ cells mL^{-1}) and OD_{680} (x) was calculated as follow: $y = 19735x - 33$ ($R^2 = 0.9999$). The inhibition ratio (IR) of cyanobacterial growth was calculated according to the following Eq. (1):

$$IR = (N_0 - N_s)/N_0 \times 100\% \quad (1)$$

where N_0 and Ns (cells mL^{-1}) represent cell density in the control and treatment groups, respectively.

2.3.3. Measurement of esterase activity

The fluorescein diacetate (FDA) assay has been used to indicate the metabolic activity of cells (Dunker et al., 2013; Xiao et al., 2010, 2011). 5 μL of 1 g L^{-1} FDA was added into 500 μL cells samples in a final concentration of 10 mg L^{-1} incubated for 15 min at room temperature in darkness. Then cells were analyzed by flow cytometer FACS Verse (BD Biosciences, Franklin Lakes, New Jersey, USA) with excitation and emission wavelength of 488 nm and 527/32 nm. As depicted by Hadjoudja et al. (2009), the changes of esterase activity exposure to P_1NA and P_2NA were showed as the decrease in the proportion of cells in the S_2 regions (normal activity status) compared with control cells. Every sample was collected 10,000 events.

2.3.4. Detection of cell membrane integrity

Cell membrane integrity was assessed by fluorescent dye PI which could interact with nucleic acids to produce red fluorescence when the cell membrane was damaged (Xiao et al., 2011). For the measurement of *M. aeruginosa* cell membrane integrity, 5 μL of PI working solution was added to 500 μL of the sample with the final concentration of 15 μM and the sample was stained for 15 min in the dark at room temperature. Fluorescence was collected through 586/42 nm with 488 nm laser. Data were expressed as stained cell percentages.

2.3.5. Detection of intracellular ROS level

The ROS level in *M. aeruginosa* was measured using the cell permeable indicator 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) according to He et al. (2016). The samples of cells were added with a final concentration of 10 μM DCFH-DA and pre-incubation at 25 °C in dark for 1 h. Sample fluorescence was measured using flow cytometer with excitation and emission wavelength of 488 nm and 527/32 nm. To prove the role of ROS in the P_1NA induced growth inhibition, the BG11 culture mediums were added with ROS scavenger ascorbic acid (0.5, 1 and 2 mM) and then adjusted to pH 7.1 with 1 M NaOH. The cells were pre-treated for 1 h and then exposed to 15 μM P_1NA for 24 h. Growth inhibition in test organism was detected.

2.3.6. Photosynthesis activity analysis

Maximum quantum yield (Fv/Fm), maximal electron transport rate (ETRmax) and light use efficiency ratio (α) were selected to represent the *M. aeruginosa* photosynthesis status. These values were measured using phyto-PAM analyzer (Walz, Effeltrich, Germany) (Maxwell and Johnson, 2000).

2.3.7. Determination of P_1NA and P_2NA concentrations and metabolites

For analyzing the concentration of P_1NA and P_2NA, 2 mL culture samples were extracted by equivalent ethyl acetate and then the extracts were dehydrated by anhydrous sodium sulfate. The extracts being diluted 20 times were then transferred to auto-sampler vials for gas chromatography–mass spectrometry (GC–MS) (Agilent 6890N/5973Inert, Santa Clara, California, USA). The GC temperature profile consisted of an initial holding time of 2 min at 40 °C followed by a gradual increase in temperature of 20 °C min^{-1}, reaching a final temperature of 320 °C, held for 24 min. The retention time for P_1NA and P_2NA were 13.5 min and 13.9 min, respectively. The standard curve was prepared and the equation for relation between peak area (y) and concentration (x) (μM) (0–1 μM) was as follows: $y = 70781x - 2533.9$ ($R^2 = 0.991$) and $y = 268031x - 5141.2$ ($R^2 = 0.997$) for P_1NA and P_2NA, respectively. The metabolites were qualitatively analyzed in *M. aeruginosa* exposed to 20 μM of P_1NA, P_2NA and control group respectively. The metabolites were determined by GC–MS, briefly as follows: 25 mL culture samples (exposure for 72 h) were adjusted to pH 2 with 1 M HCl and extracted three times with an equivalent volume of ethyl acetate. The following sample handling and determination condition were the same to the Lu et al. (2016).

2.4. Statistical analysis

Flow cytometric analysis data were analyzed using FlowJo software (Tree Star Software, San Carlos, California, USA). Data are presented in mean ± SD. One-way ANOVA followed by LSD (when homogeneity of variance) or Tamhane's T2 (when heterogeneity of variance) post hoc test was used to test for the differences between individual means among P_1NA or P_2NA treatment at different concentrations. Independent samples t-tests were used to compare the differences individual means when at the same P_1NA and P_2NA concentration. Differences were considered to be significant at $p < 0.05$. EC_{50} values were determined with probit regression. All the statistical analyses were performed using SPSS 18.0 software (IBM Corporation, Armonk, New York, USA).

3. Results and discussion

3.1. Real concentrations of P_1NA and P_2NA concentrations during experiment

In order to determine the real concentrations and stability of P_1NA and P_2NA, the concentrations in culture medium were quantitatively analyzed at 0, 24, 48 and 72 h. The results are shown in Fig. 1. For nominal concentrations of 5, 10, 15, 20 μM, the real exposure concentrations of P_1NA were 4.75 ± 0.018, 11.64 ± 1.86, 14.89 ± 0.27, 20.15 ± 0.86 μM, respectively. The real exposure concentration of P_2NA were 4.17 ± 0.11, 10.62 ± 0.48, 12.73 ± 1.46, 19.90 ± 0.17 μM, respectively. It can be found that the actually

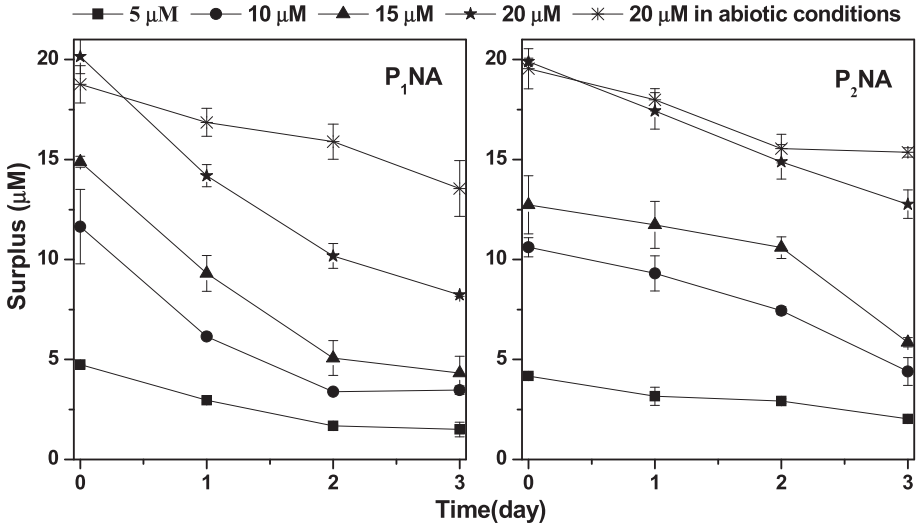

Fig. 1. Real concentrations of P_1NA and P_2NA (μM) in the culture medium, during the experimental period. Data are presented in mean ± SD.

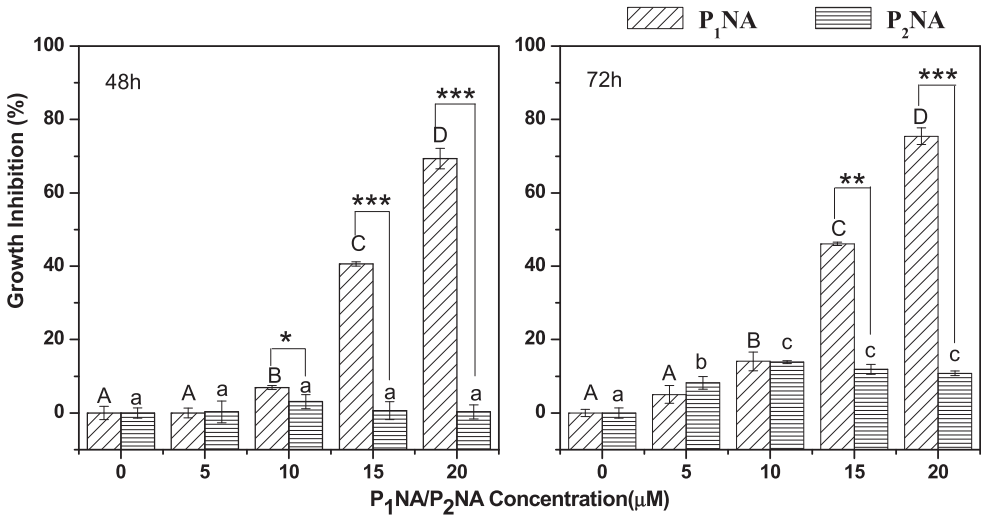

Fig. 2. Effect of P_1NA and P_2NA on growth inhibition in *M. aeruginosa*. * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) indicate significant differences between P_1NA and P_2NA. Different small letters in each figure in the same type of growth inhibition represent statistically significant differences ($p < 0.05$) compared to the corresponding controls. Data are presented in mean ± SD.

determining concentrations are close to the nominal concentrations. The average percentage error of P_1NA and P_2NA was 5.7% and 9.6% between measured concentrations and nominal values. The concentration of P_1NA and P_2NA decreased gradually over time in biotic and abiotic conditions. And the decomposition rate of P_1NA is faster than that of P_2NA. For instance, 20 μM of P_1NA and P_2NA in the culture medium were reduced by 59.2% and 35.8% within 72 h. The half-life of P_1NA was 24–50 h, while that of P_2NA was 66–120 h which was out of our experimental period calculated from the degradation patterns at 5, 10, 15, and 20 μM.

We speculated that P_1NA and P_2NA concentrations decreased possibly due to the reason that these substances can be adsorbed and metabolized by cyanobacteria or photochemically decomposed in the culture medium. Sikka et al. (1981) demonstrated that P_1NA was degraded with a half-life ranging from 4 to 11 days in water inoculated with domestic sewage and lake water, respectively. A further experiment conducted in aqueous solution, indicated that P_1NA could be directly photochemically degraded (Sikka et al., 1981). Meanwhile, on the basis of *in vitro* studies, metabolism of P_1NA and P_2NA likely occurs primarily via hydroxylation (Deutsche Forschungsgemeinschaft, 2015; Koennecker et al., 1998; Weiss et al., 2007).

3.2. Effects of P_1NA and P_2NA on *M. aeruginosa* growth

Cell growth inhibition is commonly used as a valid biomarker to evaluate the phytotoxicity on cyanobacteria as it shows. Because it is an integrating parameter showing toxicants inhibitory effects on all cellular metabolism (Dewez et al., 2008). The inhibition effects of P_1NA and P_2NA on *M. aeruginosa* growth were studied after 48 and 72 h (Fig. 2). It can be seen that when exposure to P_1NA, the growth inhibition of *M. aeruginosa* increased with an increasing concentration of P_1NA. After 48 h, the inhibition rates in the group of 5, 10, 15, 20 μM of P_1NA were 4.0%, 6.9%, 40.6%, 69.4%, respectively. However, those of P_2NA were 0.3%, 3.1%, 0.6%, 0.3%, respectively. The inhibition rates were 5.0%, 14.1%, 46.1%, 74.1%, respectively, exposure by P_1NA after 72 h. A significant dose-response and time-dependent relationship were displayed in P_1NA group, while these relationships for P_2NA were not observed in the experiment. Growth inhibition rates of P_1NA exposed to 10, 15, 20 μM were higher than that of P_2NA at 48 h, and the differences were 3.8%, 40.0%, 69.0%, respectively. It showed significant differences ($p < 0.05$) with the exposure time for 48 h in the group of 10, 15 and 20 μM, and with the exposure time for 72 h in the group of 15 and 20 μM, respectively.

The EC_{50} (48 h) value (Table S1) of P_1NA calculated from growth inhibition was 16.616 μM (3.64 mg/L), while that of P_2NA was not detected. The results showed that only P_1NA was highly toxic to *M. aeruginosa*. Koennecker et al. (1998) reported that P_1NA was low toxic to the animal but highly toxic to aquatic organisms. An EC_{50} (48 h) of 2 mg L^{-1} of P_1NA was measured for the inhibition of cell proliferation of freshwater ciliates *Tetrahymena pyriformis* (Epstein et al., 1967). LC_{50} (48 h) for young and adult *Daphnia magna* were in the range of 0.30–0.68 mg L^{-1} (Koennecker et al., 1998). The toxicity of P_1NA to *M. aeruginosa* was similar to that of protozoa, which was lower than that of cladoceran zooplankton. However, several studies have reported that P_2NA is a highly toxic substance to *C. vulgaris* (Qian et al., 2009), *M. aeruginosa* (Qian et al., 2010) and *Cylindrospermopsis raciborskii* (Liu et al., 2015). The toxicity of P_2NA may be affected by the strain of the test organism or the culture conditions. The difference needs to be furtherly studied in the future.

3.3. Impact on esterase activity

The esterase activity in *M. aeruginosa* exposed to P_1NA and P_2NA for 48 and 72 h were shown in Fig. 3. It could be found that the esterase activity significantly decreased in the group of 5 μM and 10 μM exposure to P_1NA at 48 h. And then the esterase activity drastically reduced with the increase of P_1NA concentration, it declined to 70.2% and 22.2% of the proportion of cells in the S_2 regions (normal activity status) (Hadjoudja et al., 2009) with 15 μM and 20 μM of concentrations exposure to P_1NA. The similar pattern could be found in the group exposure for 72 h, while it did not further lower the esterase activity. As opposed to P_1NA, the esterase activity was invariant as P_2NA concentrations increased at 48 and 72 h.

Esterase activity was commonly used to evaluate the metabolic status of the cell because it was a rapid and sensitive endpoint (Valiente Moro et al., 2012), and usually, a decline in enzyme activity indicated the presence of stress (Hadjoudja et al., 2009; Regel et al., 2002). Herbicide paraquat and copper could result in

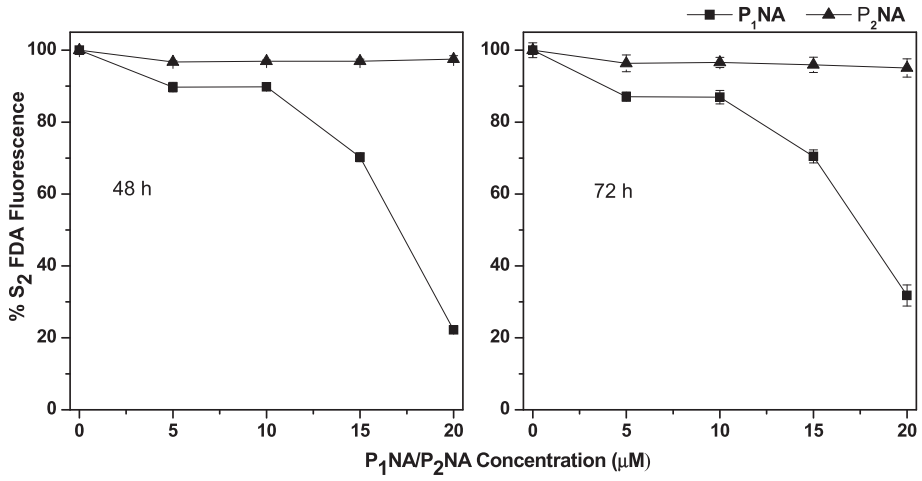

Fig. 3. Effect of P_1NA and P_2NA on esterase activity in *M. aeruginosa*. Data are presented in mean ± SD.

reducing esterase activity due to oxidative stress and showed a dose-response relationship in acute toxicity test for phytoplankton (Hadjoudja et al., 2009; Prado et al., 2012). It indicated that the decline in esterase activity exposure to P_1NA being similar to paraquat and copper. And it gave an information not only to be helpful to promptly determine the physiological state of cells but also to evaluate the toxicity of pollutants. The change of esterase activity along with growth inhibition suggested that the toxicity of P_1NA and P_2NA to *M. aeruginosa* had an obvious difference.

3.4. Inhibition of photosynthetic activity

In the present study, maximum quantum yield (Fv/Fm), maximal electron transport rate (ETRmax) and light use efficiency ratio (α) were used to estimate the efficiency of PSII in *M. aeruginosa*. PSII efficiency in *M. aeruginosa* exposure to P_1NA and P_2NA are shown in Fig. 4. The results showed that Fv/Fm has significantly decreased in the group of 15 and 20 μM P_1NA exposure for 48 and 72 h ($p < 0.05$) (Fig. 3A and B). It was different when exposure to P_2NA. At 48 h, Fv/Fm was significantly decreased in the group of 10, 15 and 20 μM P_2NA ($p < 0.05$). But every concentration of the P_2NA treatment group did not have any differences at 72 h ($p > 0.05$) which was consistent with the trend of growth inhibition. There was a significant difference between P_1NA and P_2NA in the group of 15 and 20 μM at 48 and 72 h. The effects on ETRmax and α are shown in Fig. 4C, D and E, F, respectively. P_1NA treatment led to a significant decrease in ETRmax or α values at high concentration, while exposure of P_2NA slightly reduced them. It was much similar to the change of Fv/Fm values.

The P_1NA caused a severe toxicity on PSII of the cyanobacteria *M. aeruginosa*, as introduced above. These results were consistent with the growth and esterase activity of *M. aeruginosa*. It is known that the value of Fv/Fm characterizes the content of photochemically active centers of PSII and indicates the current balance between processes of light destruction and the reparation of PSII (Brack and Frank, 1998). Similarly, some kinds of exogenous substances such as tetracycline and natural flavonoids were found to inhibit growth and reduce Fv/Fm values in *M. aeruginosa* and *Selenastrum capricornutum* (Huang et al., 2015; Yang et al., 2013). Allelochemicals identified from *Myriophyllum spicatum*, including pyrogallic acid, gallic acid, ellagic acid and (+)-catechin caused significant reductions of PSII efficiency and whole electron transport chain activities in *M. aeruginosa* (Zhu et al., 2010). The decrease of the ETRmax suggested that stress resulting from exposure to P_1NA and P_2NA had inhibitory effects on the donor side of PSII in *M. aeruginosa*. Blockage of electron transfer chain accompanied by a decrease in Fv/Fm (Han et al., 2008; Liu et al., 2015). In *M. aeruginosa*, the initial effect concentrations showing a significant difference between P_1NA and P_2NA for ETRmax (5 μM) was lower than that for Fv/Fm (10 μM) for 48 h. The disparity between the responses of Fv/Fm and ETRmax reflects inhibition downstream of PS II, suggesting that the down-regulation of PS II electron transport is a result of inhibition of Calvin cycle enzymes and Rubisco (Han et al., 2008; Yu et al., 2002). But in general, though P_2NA can slightly weaken the photosynthesis of *M. aeruginosa*, it is no doubt that the toxicity is different between P_1NA and P_2NA in *M. aeruginosa*.

3.5. Effect on cell membrane integrity

The percentage of PI-stained cells of *M. aeruginosa* exposure to P_1NA and P_2NA are presented in Fig. 5. After 48 h, percentage of cells stained by PI exposed to 5, 10, 15, 20 μM, of P_1NA were 7.0%, 7.6%, 21.3%, 61.4%, respectively. However, that of P_2NA were 3.2%, 3.2%, 3.4%, 3.3%, respectively. It showed that as P_1NA concentration increased, the PI positive cell percentage was increased in a dose-response relationship and it had a significant difference in every P_1NA concentration being tested compared to the control ($p < 0.05$). However, there was no change in the integrity of the cell membrane in *M. aeruginosa* when exposure to P_2NA ($P > 0.05$). And it all showed a significant difference in same exposure concentration between P_1NA and P_2NA ($p < 0.05$).

Cells stained by PI is a recognized method being used to probe cell membrane integrity for the assessment of the environmental impact of toxic contaminants in aquatic systems. Herbicide paraquat and allelochemical tannic acid have been reported to lead damages to the membrane integrity of phytoplankton cells indicating a reduction in the percentage of viable cells exposed to that agent (Eigemann et al., 2013; Prado et al., 2009). Studies reported that aquatic plants such as *E. crassipes* (Sultankhodzhaev and Tadzhibaev, 1976; Sun et al., 1993), *Elodea nuttallii*, *Hydrilla verticillata* and *Vallisneria spiralis* (Gao, 2010) could secrete P_1NA and P_2NA, which showed high allelopathic activity. Xiao et al. (2010) indicated that assay of cell membrane integrity was a novel and fast method to determine whether the antialgal agents were algicidal (killing algae) or algistatic (preventing algal growth). This paper suggested that P_1NA has the nature of killing cyanobacteria together with growth assays and cell membrane integrity tests. The

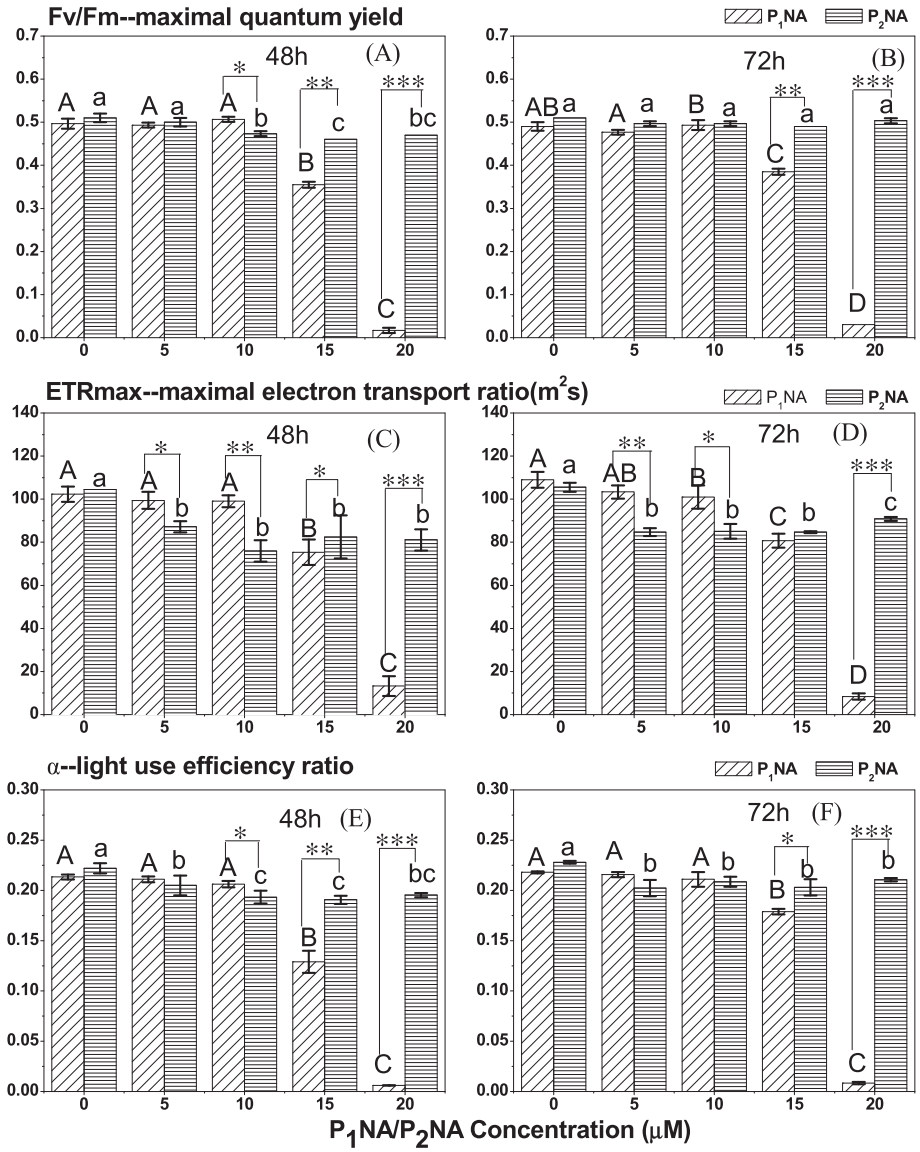

Fig. 4. Effects of P_1NA and P_2NA on Fv/Fm (A, B), ETRmax (C, D) and α (E, F) in *M. aeruginosa*. (F). * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) indicate significant differences at the same concentration of P_1NA and P_2NA. Different small letters in each figure in the same type of Fv/Fm, ETRmax or α represent statistically significant differences ($p < 0.05$) compared to the corresponding controls without P_1NA or P_2NA.

studies indicated that P_1NA is the allelochemical which shows higher inhibitory activities to cyanobacteria than that of P_2NA.

A comparison of the sensitivity tested by different endpoints including growth inhibition, esterase activity, cell membrane integrity and the efficiency of photosynthesis was performed. And the EC_{50} values of P_1NA were given in Table S1. The EC_{50} values based on growth inhibition, esterase activity, cell membrane integrity and Fv/Fm were 16.616, 17.445, 16.099, 19.604 μM, respectively, which were not far from each other. These results suggested that the parameters being tested were effective endpoints in acute toxicity test. However, EC_{50} values of P_2NA was not detected in same conditions in this study. All results shown above indicated that the acute toxicity test of P_1NA and P_2NA in *M. aeruginosa* exhibited significant difference.

3.6. Toxic mechanism of P_1NA

Oxidative damage induced by ROS has been proved to be the reason resulting in the toxic effect of chemical toxicants such as anthraquinone derivative (Schrader et al., 2005) and pyrogallic acid (Lu et al., 2016) to phytoplankton. To reveal the possible mechanism of the different toxicity between P_1NA and P_2NA, the intracellular ROS level was measured. From Fig. 6, the remarkable increase in intracellular ROS levels with the increase of P_1NA concentration was observed after 48 h exposure ($p < 0.05$). The ROS level in the group of 5, 10, 15, 20 μM P_1NA were 1.31, 1.75, 2.46, 2.83 times higher than that of control, respectively. However, a significant dose-response relationship was not found in the group exposure to P_2NA, in which slight lower level than the control was observed.

ROS including $O_2^{\bullet -}$, H_2O_2 and •OH are produced by the normal metabolic process and maintain a dynamic balance with the antioxidant system in organisms. The overproduction of ROS in phytoplankton was in response to toxicants with redox-cycling property and adverse environmental stresses (Wang et al., 2012; Yang et al., 2011). It was reported that the acute increase of ROS induced by gramine could be a potential cause of the growth inhibition of cyanobacteria (Hong et al., 2009). In addition, it has been also demonstrated that the overproduction of ROS affected cell

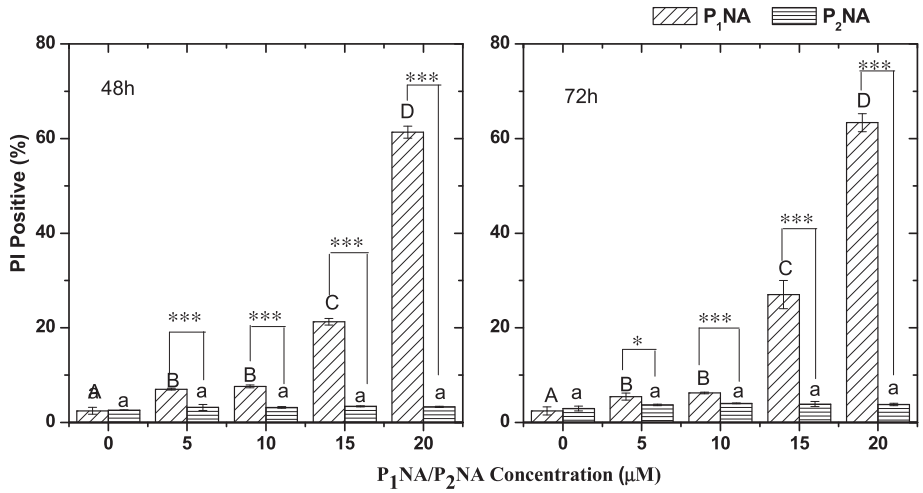

Fig. 5. Effect of P_1NA and P_2NA on cell membrane integrity in *M. aeruginosa*. * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) indicate significant differences between P_1NA and P_2NA. Different small letters in each figure in the same type of growth inhibition represent statistically significant differences ($p < 0.05$) compared to the corresponding controls. Data are presented in mean ± SD.

growth or proliferation. For example, Wang et al. (2011) reported that allelochemicals (+)-catechin and pyrogallic acid can induce the generation of intracellular ROS to inhibit the growth of two freshwater algae.

As is known that high level of ROS can directly damage proteins, nucleic acids, and cell membrane (Yu et al., 2007), finally leading to growth inhibition and cell death. In order to evaluate the effects of ROS level on the growth inhibition, the decrease of esterase activity, Fv/Fm, and cell membrane integrity, the relevance between ROS and these four parameters was analyzed. The regression curve and correlation coefficients (R^2) were given in Table S2. The R^2 between ROS and these four parameters mentioned above were 0.892, 0.781, 0.694, 0.694, respectively. It suggested that increased ROS level was the major mechanism contributing to the growth inhibition of *M. aeruginosa* exposure to P_1NA under the experimental conditions. In addition, the regression analysis was done for the relationships between the growth inhibition effect and ROS, esterase activity, Fv/Fm and cell membrane integrity.

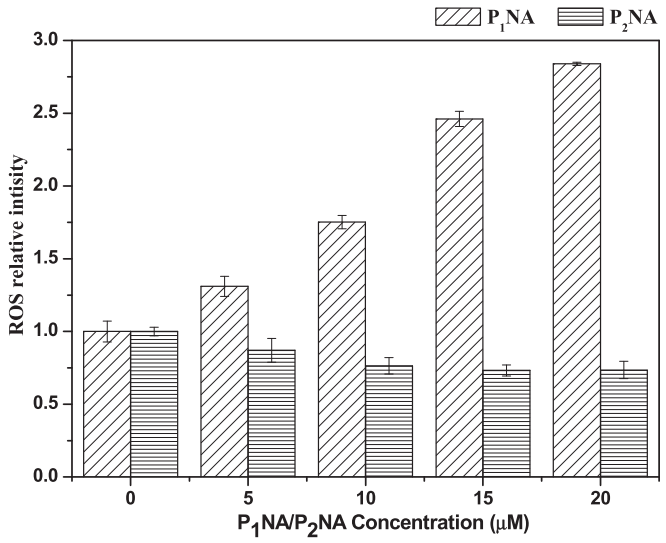

Fig. 6. Intracellular ROS level in *M. aeruginosa* exposure to P_1NA and P_2NA for 48 h. Relative ROS level was reflected by the mean fluorescence intensity of DCF. Data are presented in mean ± SD.

To furtherly verify the role of ROS in the growth inhibition of *M. aeruginosa* exposed to P_1NA, the protective effect of ascorbic acid, a kind of free radical scavenger, was investigated. As illustrated in Fig. S1, growth inhibition induced by 15 μM P_1NA in *M. aeruginosa* was significantly alleviated after the treatments of ascorbic acid. Ascorbic acid was the widely and water-soluble antioxidant for the prevention or minimization of damages caused by ROS in plants (Khan and Ashraf, 2008). Lu et al. (2016) demonstrated that ascorbic acid could decrease the DNA strand breaks of *M. aeruginosa* exposed to pyrogallic acid. The result of ROS scavenger experiments demonstrated that the excessive formation of ROS induced by P_1NA was the main cause leading to growth inhibition in *M. aeruginosa*.

In vitro studies have demonstrated that P_1NA was primarily metabolized via hydroxylation (Koennecker et al., 1998) to form Mono- and dihydroxy-metabolites (Sikka et al., 1981; Wolff, 1992). However, only the monohydroxy-metabolites 6-hydroxy-N-phenyl-2-naphthylamine and 4′-hydroxy-N-phenyl-2-naphthylamine were detected after exposure with P_2NA (Deutsche Forschungsgemeinschaft, 2015; Weiss et al., 2007). Dihydroxy-metabolites can form quinone while monohydroxy-metabolites not. As summarized previously (O'brien, 1991), oxidative stress arises when the quinone is reduced by reductases to a semiquinone radical which reduces oxygen to superoxide radicals and reforms the quinone. Wang et al. (2011) reported that polyphenolic allelochemicals such as (+)-catechin and pyrogallic acid could elevate cellular ROS level through futile redox cycles, which amplify the generation of $O_2^{\bullet -}$ with the expense of NAD(P)H and greatly enhance the toxicity to the target organisms. The results suggested that the generation of ROS was related to the biotransformation metabolites of P_1NA. Therefore, the metabolites of P_1NA and P_2NA in *M. aeruginosa* were detected by GC/MS. 1,4-naphthoquinone was found in *M. aeruginosa* exposure to P_1NA, while it was not detected in P_2NA and control groups (Fig. S2). We speculated P_1NA metabolized to form 1,4-dihydroxynaphthalene firstly. It was similar to (+)-catechin and pyrogallic acid which may cause autoxidation or enzymatic oxidation to easily form semiquinone radical in the target organism. The reactive semiquinone radical is able to react with O_2 to produce o-quinone form of 1,4-dihydroxynaphthalene accompanying the production of $O_2^{\bullet -}$. Dismutation of $O_2^{\bullet -}$ produces H_2O_2, and then H_2O_2 can be reduced via the Fenton-type reaction in the presence of transition

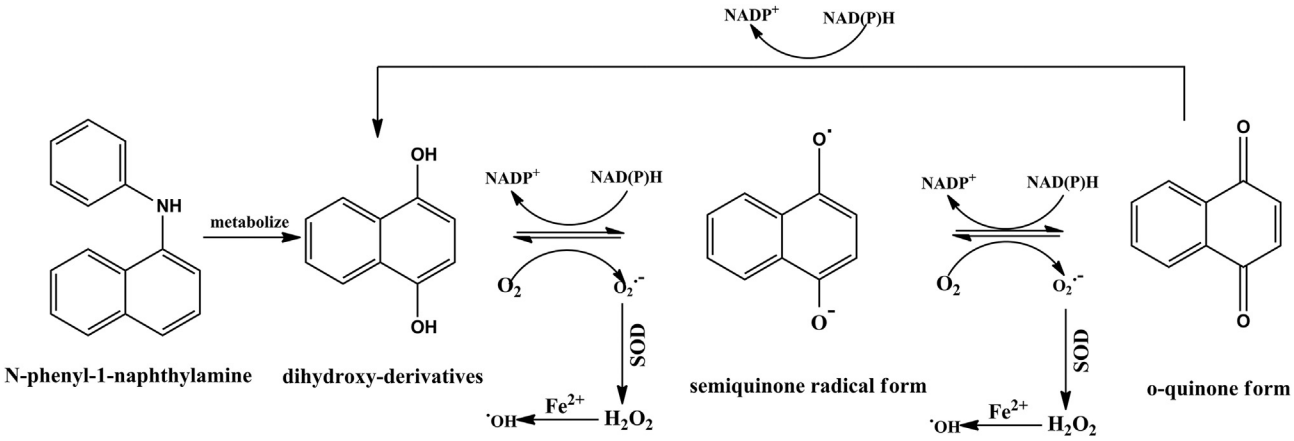

Fig. 7. A possible pathway of ROS production induced by P_1NA in *M. aeruginosa*.

metals to form the ·OH. Therefore, it is reasonable to expect that the metabolites of P_1NA may undergo futile redox cycling in the presence of intracellular reductants such as NAD(P)H (O'brien, 1991) and induce ROS production in *M. aeruginosa*. A possible pathway of ROS generation in *M. aeruginosa* induced by P_1NA could be proposed as shown in Fig. 7. In brief, it was the first time to demonstrate that P_1NA exhibited remarkably stronger growth inhibition effects in *M. aeruginosa* than its isomer P_2NA. The different property in inducing excessive ROS between P_1NA and P_2NA was the important reason to exhibit different toxicity in *M. aeruginosa*. The formation of 1,4-naphthoquinone was the key factor to generate excessive ROS.

4. Conclusion

The results of comparing the toxicity of P_1NA and P_2NA indicated that only P_1NA was highly toxic to *M. aeruginosa*. The 48 h EC_{50} values based on growth inhibition were 16.616 μM, while that of P_2NA was not detected in same conditions. The intracellular ROS levels were significantly increased in *M. aeruginosa* exposure to P_1NA, while it was not found in the group of P_2NA. Attention should be paid to the influence of P_1NA on other aquatic organisms and aquatic ecosystem. In the research field of allelopathy, the toxic effect of P_1NA on phytoplankton should be investigated as the main object compared with P_2NA.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.02.110.

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